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Luciferase activity under direct ligand-dependent control of a muscarinic acetylcholine receptor

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Abstract

Background: Controlling enzyme activity by ligand binding to a regulatory domain of choice may have many applications e.g. as biosensors and as tools in regulating cellular functions. However, until now only a small number of ligand-binding domains have been successfully linked to enzyme activity. G protein-coupled receptors (GPCR) are capable of recognizing an extraordinary structural variety of extracellular signals including inorganic and organic molecules. Ligand binding to GPCR results in conformational changes involving the transmembrane helices. Here, we assessed whether ligand-induced conformational changes within the GPCR helix bundle can be utilized to control the activity of an integrated enzyme.

Results: As a proof of principle, we inserted the luciferase amino acid sequence into the third intracellular loop of the M₃ muscarinic acetylcholine receptor. This fusion protein retained both receptor and enzyme function. Receptor blockers slightly but significantly reduced enzyme activity. By successive deletion mutagenesis the enzyme activity was optimally coupled to ligand-induced conformational helix movements.

Conclusion: Our results demonstrate that in engineered GPCR-enzyme chimeras, intracellular enzyme activity can be directly controlled by a GPCR serving as the extracellular ligand-binding domain.

Background

Synthetic protein biosensors are typically designed by fusing a target-binding domain to an easily assayed reporter protein. The ligand-binding domain is often derived from a specific receptor protein. Most biosensors are complementation systems where enzyme or fluorophore activity is reconstituted from non-functional domains secondary to receptor dimerization or conformational changes upon ligand binding [1,2]. However, the repertoires of both,

well characterized ligand-binding and reporter domains that are suitable for the design of complementation systems are rather small. A modular system that allows arbitrary combination of ligand-binding and reporter domains would thus be most desirable. There are a few examples of properly constructed receptor-enzyme chimeras which allow for the transduction of ligand-induced conformational changes within the receptor to the reporter protein and allosteric modulation of its proper-

ties [3-5]. Coupling of ligand-induced conformational changes to reporter protein function appears to be the general bottleneck in designing such biosensors.

Among the different families of transmembrane receptors, G protein-coupled receptors (GPCR) form the largest receptor superfamily comprising over 1000 members within several vertebrate genomes [6-8]. Signals as multi-form as light, small molecules including ions, amines, amino acids, peptides, lipids, sugars, as well as large proteins are recognized by receptors of this class [9,10]. Upon agonist binding to the extracellular portion of GPCR, conformational changes of the transmembrane helix (TMH) bundle and intracellular loop (ICL) portions lead to G-protein activation [11-16]. Conformational changes of the TMH bundle are not only observed after agonist binding but also after binding of inverse agonists that do not result in G-protein activation [17-19]. In addition, GPCR can be modified by site-directed mutagenesis to respond to biologically inert compounds instead of their native agonists [20,21]. The combination of these properties favours GPCR as ideal ligand-binding modules in hybrid biosensors.

In a number of studies, enzymes, such as luciferases, galactosidase, alkaline phosphatase and peroxidase, as well as fluorescent proteins (e.g. YFP) have been integrated into GPCR. In these GPCR fusion proteins, the enzyme or fluorescence activities were used as reporter assay to monitor intracellular receptor trafficking [22,23] and, in fluorescence and bioluminescence resonance energy transfer (FRET/BRET) approaches, to monitor GPCR-protein interactions [24-27]. Recently, the activity of ion channels coupled to GPCR was modulated upon ligand binding, demonstrating that GPCR are suitable binding domains for biosensors [28].

Here, we report the allosteric modulation of enzyme activity upon ligand binding to a GPCR-enzyme chimera. Using the M_3 muscarinic acetylcholine receptor (M_3R) and luciferase as model proteins, we provide a proof of concept that through rigid-body movement of the TMH bundle, extracellular signals may be transduced onto an enzyme that is integrated into the cytosolic portion of a GPCR, thus changing enzyme activity.

Results and discussion

Functional integration of luciferase into the M_3R

To generate a biosensor in which enzyme activity is allosterically controlled by a binding domain of a GPCR, we replaced a 195-amino acid segment of the third ICL (ICL3) of M_3R (amino acid positions 274-469) with the sequence of luciferase from *Photinus pyralis* (referred to as M_3R -luci, Figures 1 and 2). Previous studies in mammalian expression systems have shown that in M_3R , the

removal of the central part of ICL3 has no significant effect on receptor function [29-31]. As shown in Figure 3 and summarized in Table 1, M_3R -luci expressed in COS-7 cells was delivered to the cell surface and displayed both luciferase activity (Figure 3A) and carbachol (CCh)-induced inositol phosphate (IP) formation (Figure 3B). However, enzyme activity was not influenced by application of the agonist CCh but we noted a significant reduction (15%, $p < 0.001$) in luciferase activity in the presence of the inverse agonist atropine (Figure 3A, Table 1). The effect of atropine was not found when luciferase was inserted into the second intracellular loop (ICL2) or fused to a truncated M_3R (constructs #21 and #22, Figure 2, Table 1) and in several other controls (luciferase alone, V_2 vasopressin receptor (V_2R)) [see Additional file 1] [see Additional file 2] [see Additional file 3].

Optimization of the M_3R -luciferase fusion protein

Next, we systematically deleted portions of ICL3 flanking the enzyme (Figure 2) to improve coupling of atropine-induced conformational changes to enzyme activity. Successive N-terminal shortening of ICL3 (constructs #1-#5) progressively reduced cell surface expression, ligand-induced IP formation and luciferase activity but atropine failed to modulate luciferase activity. Likewise, the C-terminal part of ICL3 was shortened (constructs #6-#8). Constructs #6 and #8 were properly delivered to the cell surface and displayed high luciferase activity. Strikingly, both chimeras showed a significant reduction in enzyme activity up to ~40% (construct #8, Table 1) upon atropine binding. G-protein signalling was retained in construct #6 but abolished in construct #8, presumably because the deleted C-terminal part of ICL3 is involved in G-protein coupling [32]. The combination of N- and C-terminal shortening (construct #9) was not advantageous.

We then tested whether N- or C-terminal truncation of the luciferase insert improved coupling to conformational changes of the receptor. Only deletion of the very N-terminal two amino acid residues (construct #10) had a significant effect on atropine-induced reduction of the enzyme activity (Table 1). Combination with ICL3 shortening (construct #11) did not further improve allosteric modulation of the enzyme activity by atropine. In agreement with previous studies [33], further removal of the N terminus abolished enzyme activity completely (Table 1, constructs #12-#18) probably because of destruction of the functionally relevant N-terminal domain. The crystal structure of *Photinus pyralis* luciferase demonstrates that N-terminal and C-terminal domains form the active site [34]. C-terminal truncation of the enzyme did not result in significantly reduced enzyme activity after atropine application, neither alone (construct #19) nor in combination with ICL3 truncation (construct #20). Preliminary studies with fusion proteins of other GPCR and reporter

Table 1: Functional properties of wild-type M₃R and M₃R-luciferase fusion proteins.

mutant	luciferase activity			IP accumulation (fold over GFP basal)		Cell surface expression (% of M ₃ R-luci)
	basal (% of M ₃ R-luci)	100 μM CCh (% of basal activity)	100 μM atropine (% of basal activity)	basal	100 μM CCh	
M ₃ R	0.08 ± 0.03 (3)	-	-	2.93 ± 0.37	12.1 ± 2.0	229 ± 13
M ₃ R-luci	100 (31)	105 ± 5	84.6 ± 3.5**	1.60 ± 0.15	10.6 ± 0.2	100
#1	71.2 ± 0.7 (3)	103 ± 9	74.8 ± 9.3	1.40 ± 0.12	9.60 ± 0.45	74.7 ± 5.3
#2	42.8 ± 4.8 (3)	116 ± 5	93.0 ± 4.2	1.20 ± 0.12	4.37 ± 0.09	56.1 ± 4.1
#3	29.8 ± 3.0 (4)	91.0 ± 11.0	95.2 ± 10.9	0.93 ± 0.03	1.57 ± 0.12	32.8 ± 5.3
#4	16.6 ± 3.0 (3)	110 ± 3.8	104 ± 6.8	0.97 ± 0.03	1.10 ± 0.06	13.5 ± 0.7
#5	11.1 ± 1.5 (3)	104 ± 10	96.0 ± 21.2	0.97 ± 0.09	1.10 ± 0.06	3.9 ± 0.9
#6	95.7 ± 5.5 (20)	94.0 ± 7.1	75.7 ± 5.2**	1.27 ± 0.09	9.50 ± 0.61	105 ± 3.2
#7	42.0 ± 3.7 (11)	95.9 ± 7.5	93.8 ± 5.9	1.00 ± 0.10	3.27 ± 0.17	35.8 ± 4.2
#8	62.6 ± 5.2 (22)	98.5 ± 4.7	60.2 ± 2.8**	0.87 ± 0.03	1.37 ± 0.09	114 ± 7
#9	25.9 ± 3.7 (5)	98.4 ± 8.7	87.3 ± 8.6	1.03 ± 0.09	1.43 ± 0.12	27.3 ± 3.5
#10	37.0 ± 4.0 (5)	95.2 ± 7.3	76.4 ± 4.1*	1.53 ± 0.17	9.67 ± 0.78	31.6 ± 4.4
#11	30.3 ± 3.9 (5)	83.4 ± 7.3	73.8 ± 3.1*	1.40 ± 0.06	8.33 ± 0.54	28.9 ± 4.8
#12	37.2 ± 6.8 (4)	111 ± 17	78.6 ± 10.1	1.43 ± 0.17	9.90 ± 0.70	56.6 ± 7.8
#13	27.8 ± 6.7 (3)	80.2 ± 8.3	76.3 ± 8.5	1.13 ± 0.09	8.87 ± 1.01	36.7 ± 11.6
#14	42.0 ± 11.3 (4)	106 ± 10	85.5 ± 6.4	1.50 ± 0.12	9.57 ± 0.95	39.5 ± 4.3
#15	31.8 ± 6.5 (4)	102 ± 5	84.3 ± 7.4	1.10 ± 0.10	8.80 ± 0.95	25.5 ± 2.5
#16	0.4 ± 0.1 (3)	-	-	1.07 ± 0.09	5.23 ± 0.41	14.6 ± 3.8
#17	0.4 ± 0.1 (3)	-	-	1.00 ± 0.06	4.40 ± 0.45	12.3 ± 5.7
#18	0.5 ± 0.3 (3)	-	-	0.87 ± 0.07	1.80 ± 0.15	5.9 ± 2.5
#19	69.3 ± 7.6 (4)	92.3 ± 16.1	66.4 ± 9.4	1.47 ± 0.19	10.9 ± 1.5	76.6 ± 12.6
#20	19.5 ± 1.3 (4)	99.6 ± 4.5	91.8 ± 9.0	0.93 ± 0.09	4.13 ± 0.12	35.2 ± 2.9
#21	17.8 ± 2.4 (7)	99.5 ± 3.3	96.1 ± 4.1	1.07 ± 0.19	1.03 ± 0.07	6.6 ± 1.6
#22	24.3 ± 10.9 (5)	97.4 ± 4.2	97.2 ± 9.1	1.03 ± 0.07	0.93 ± 0.09	0.6 ± 0.5

To evaluate the functional properties of M₃R-enzyme chimeras, luciferase activities, measured in the absence (basal activity) and presence of the indicated ligands, were determined (see Methods). Basal activity is given as percentage of M₃R-luci (411,043 ± 95,142 AU). Activity in the presence of ligands is given as percentage of the activity of the individual constructs. Data are presented as means ± S.E.M. of the indicated number of experiments, each carried out in triplicate. For measurement of G_q/phospholipase C activation IP accumulation assays were performed as described under Methods. Basal IP values were determined in control (GFP)-transfected COS-7 cells (716 ± 81 cpm/well). Data (three independent assays performed in triplicate) are given as means ± S.E.M. fold over GFP-transfected IP values. Cell surface expression was assayed with indirect cell surface ELISA. Specific optical density (OD) readings (OD value of HA-tagged construct minus OD value of GFP-transfected cells) are given as a percentage of M₃R-luci. The nonspecific OD (GFP) was 0.156 ± 0.02 (set as 0%) and the OD value of M₃R-luci was 0.66 ± 0.06 (set as 100%). ELISA data are given as means ± S.E.M. of three independent experiments, each carried out in triplicate.

Significant reduction of the luciferase activity (*p < 0.01; **p < 0.001)

proteins indicate that integration and optimization is required for every individual biosensor (data not shown).

Allosteric modulation of luciferase activity by M₃R blockers

Finally, we investigated the specificity and potency of other M₃R blockers in M₃R-luci constructs. Scopolamine and butylscopolamine, both are inverse agonists at M₃R, were most efficient in reducing luciferase activity in construct #8 [see Additional file 4]. IC₅₀ values for atropine and scopolamine were 6.6 ± 2.3 nM and 2.1 ± 0.4 nM, respectively (Figure 4). In agreement with functional studies at the wild-type M₃R [35], butylscopolamine was less potent in luciferase inhibition (IC₅₀ value: 1.7 ± 0.5 μM, Figure 4). This indicates an unchanged pharmacology of the ligand-binding domain within the M₃R-luciferase fusion protein.

From the constructs tested, construct #8 met best the criteria of a biosensor, including nanomolar ligand sensitivity, high cell surface expression, and reduced G protein-coupling (Figures 3 and 4, Table 1). In this construct, structural optimization was successfully used to enhance coupling of ligand-induced rigid body movement of the TMH to structural changes within the luciferase domain. As this leads to a reduction in luciferase activity, one can speculate that the underlying conformational change rearranges the orientation of the N- and C-terminal domains within the luciferase molecule that form its active centre. In addition, our data support previous findings [18,19] that structural changes upon inverse agonist binding are different from those induced by agonists. Following CCh stimulation, fusion protein #6 still activated the G_q/phospholipase C pathway but CCh had no influence on luciferase activity (Table 1). However, atropine reduced

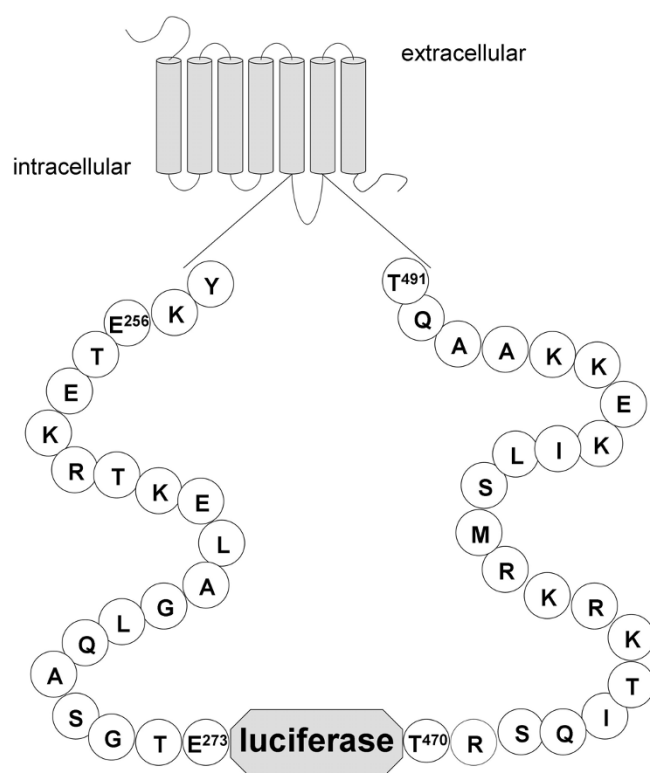


Figure 1
Schematic presentation of the M₃R-luciferase fusion construct. Outline of M₃R-luci with detailed illustration of ICL3 containing the luciferase sequence. Details of all fusion constructs are given in Figure 2.

luciferase activity by 25% in this construct. It is interesting to note that insertion of luciferase, which itself is bigger than the receptor protein, into the cytoplasmic surface of the GPCR still allows for efficient G-protein coupling. This implicates a rather small interaction site between the receptor and the G protein.

Conclusion

By integration of luciferase into ICL3 of a GPCR and successive optimization, we generated a fusion protein in which the enzyme activity is allosterically modulated by ligand binding to the GPCR in the nanomolar range. Thus, we demonstrate that GPCR are suitable as modular ligand binding domains capable of transducing the signal through rigid-body movement of TMH onto an intracellularly integrated enzyme. Artificial GPCR-enzyme chimeras may have applications beyond biosensing, for example, in ligand-dependent control of metabolic pathways and as molecular tools to study conformational changes in GPCR. Our study may also encourage the generation of GPCR fusion proteins in which other proteins or protein domains such as fluorescent proteins and SH3 domains

are allosterically regulated to modulate fluorescence properties and protein-protein interactions, respectively.

Methods

Materials

The agonist carbamylcholine chloride (carbachol, CCh) and the inverse agonists [36] atropine sulfate, scopolamine hydrochloride, and n-butyl scopolamine bromide were obtained from Sigma. Substances were solved in water and stock solutions (100 mM) were aliquoted and stored at -20°C. Aliquots were thawed only once. All restriction enzymes for cloning purposes were purchased from NEB and primers [see Additional file 5] were synthesized by Invitrogen.

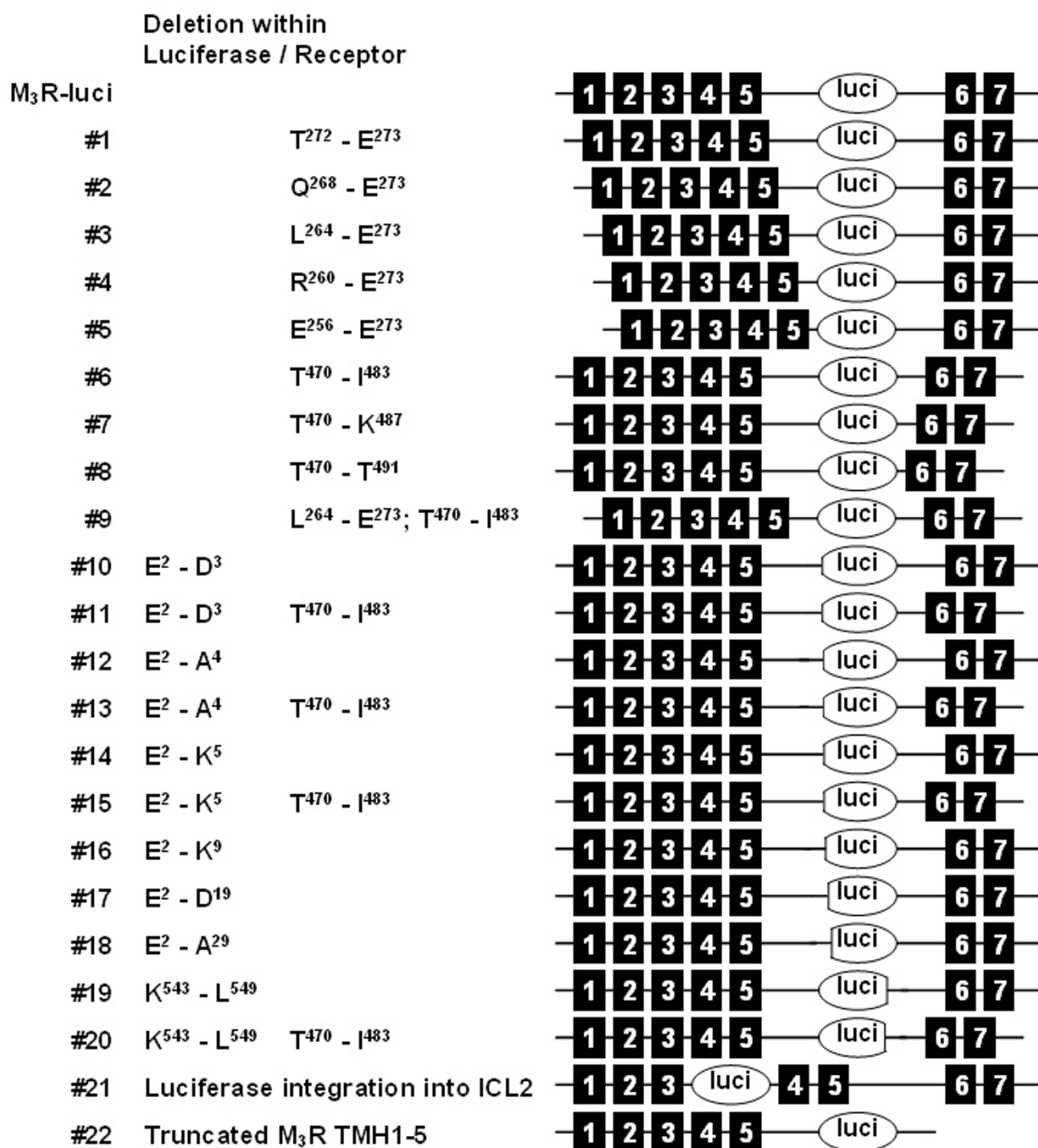
Construction of plasmids and mutants of mAChR

All mutations were introduced into the rat M₃R [30]. In this rat M₃R construct the central part of ICL3 was removed and the N- and C terminus contained an HA- and a Flag-tag, respectively. These modifications were previously demonstrated to have no significant effect on receptor function [29-31].

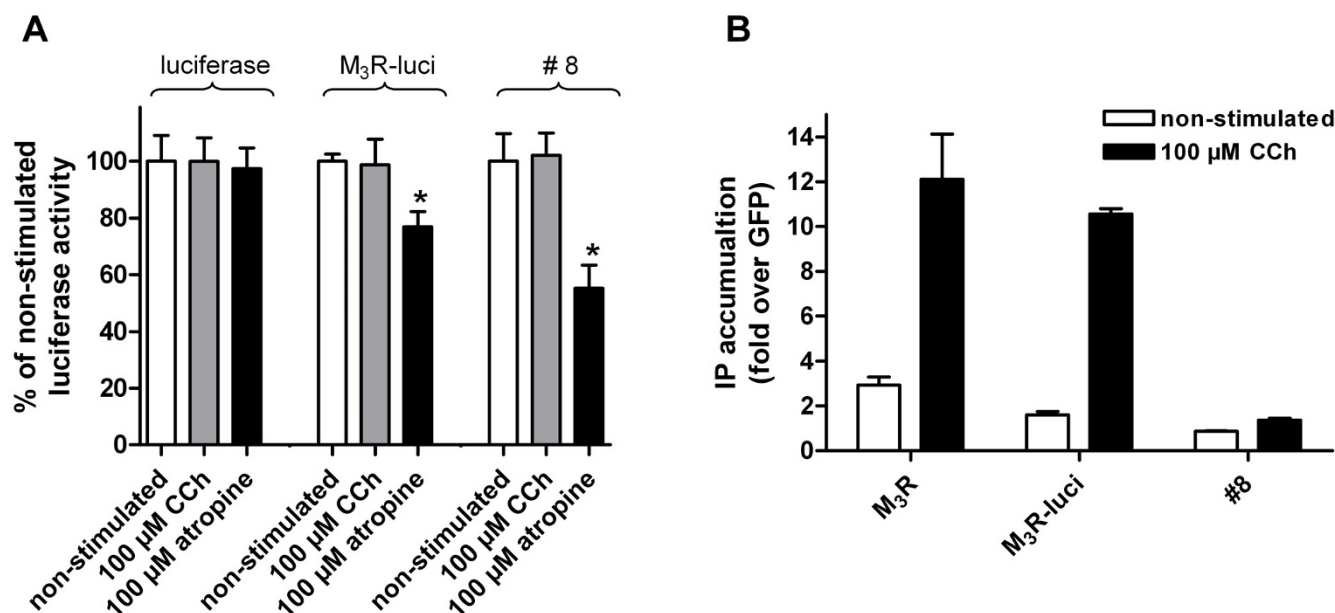
The cDNA from *Photinus pyralis* luciferase (without start and stop codons) was amplified and introduced into the ICL3 of M₃R by a PCR-based strategy. Luciferase cDNA was 5' and 3' flanked by *SpeI* sites which allowed for systematic and convenient shortening of ICL3 during optimization experiments. All other mutations were introduced by PCR-based and fragment replacement strategies into M₃R-luci (Figure 1 and 2). For control purposes, several additional constructs were generated. Here, luciferase was integrated into ICL2 (Figure 2, construct #21) or C-terminally fused to M₃R that was truncated in ICL3 (Figure 2, construct #22). Moreover, luciferase was also integrated into V₂R lacking the central portion of ICL3 (positions R²⁴³ – T²⁵³). The identity of all constructs and the correctness of all PCR-derived sequences were confirmed by restriction analysis and DNA sequencing.

COS-7 cell culture and transfection

COS-7 cells were grown in Dulbecco's modified Eagle medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidified 7% CO₂ incubator. Rotifect (Roth) was used for transient transfection. For measurement of luciferase activity, 6×10^5 cells were seeded into 6-cm dishes and transfected with 2 µg of plasmid DNA. Twenty-four hours after transfection, cells were split into white 96-well culture plates (PerkinElmer) at 30,000 cells per well. IP formation was determined in 12-well plates (1.5×10^5 cells/well transfected with 0.5 µg of plasmid DNA/well). For the measurement of cell surface expression, cells were seeded into 48-well plates (3.5

**Figure 2**

Fusion constructs and deletion mutants used in the study. Mutants were generated by successive deletion of N- and C-terminal domains of the enzyme and loop portions of the receptor (deleted portions are given). For control experiments luciferase was also integrated into ICL2 (#21) and fused to a truncated M₃R only consisting of TMH1-5 (#22).

**Figure 3**

Functional integration of luciferase into the M₃ muscarinic acetylcholine receptor. A) COS-7 cells were transfected with luciferase construct, M₃R-luci and construct #8 and luciferase activity was determined after incubation without and with the indicated ligands. The luminescences without ligands were $1,905,212 \pm 172,463$ AU (luciferase), $196,512 \pm 4,942$ AU (M₃R-luci) and $100,473 \pm 9,770$ AU (#8). All data are given as means \pm SEM of three independent experiments each performed in triplicate. B) Basal and CCh-induced IP formation was determined in COS-7 cells transfected with the wild-type M₃R, M₃R-luci and construct #8. All data are given as means \pm SEM of three independent experiments each performed in triplicate.

$\times 10^4$ cells/well) and transfected with 0.2 μ g of plasmid DNA/well.

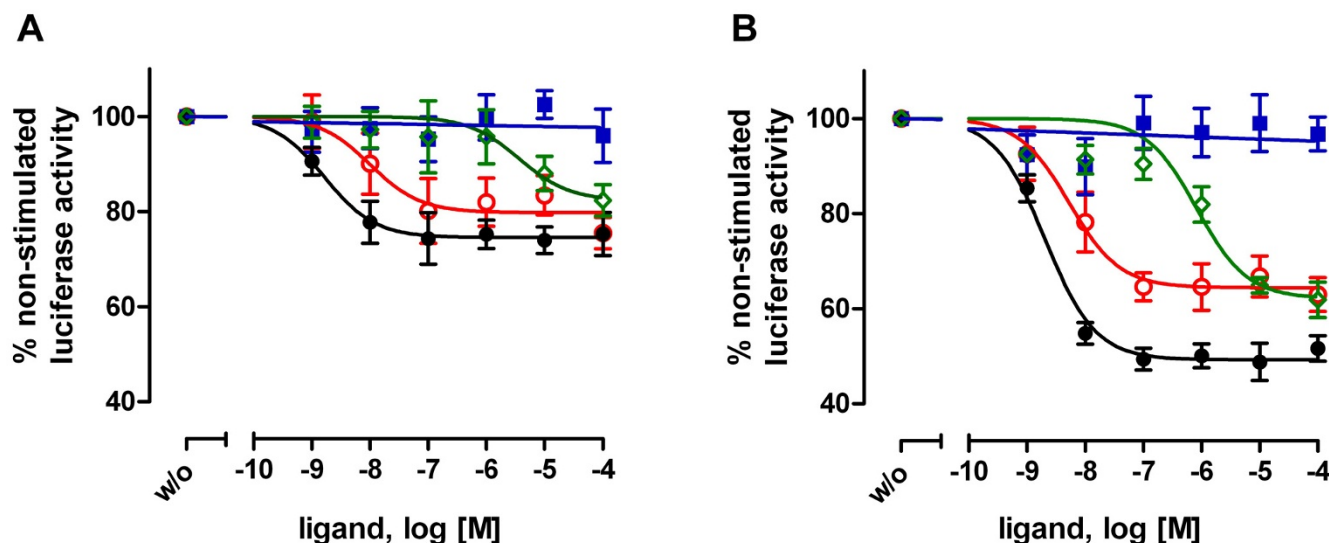
Functional assays

To measure IP formation transfected COS-7 cells were incubated with 2 μ Ci/ml of *myo*-³H-inositol (18.6 Ci/mmol, PerkinElmer) for 18 h. Thereafter, cells were washed once with serum-free DMEM containing 10 mM LiCl, followed by incubation with ligands for 30 min at 37°C. Intracellular IP levels were determined by anion-exchange chromatography as described previously [37].

The luciferase activity assay was performed with a luciferase activity detection system based on the method described by van Leeuwen et al. [38]. Briefly, after incubation with the ligand in DMEM for 20 min at 37°C, cells were washed with ice-cold PBS. Cell lysis was performed on ice by adding 20 μ l of lysis buffer (77 mM K₂HPO₄, 23 mM KH₂PO₄, 0.2% Triton X-100, 1 mM DTT, pH 7.8) per well. The lysate was mixed with 100 μ l of luciferase buffer (20 mM tricine, 2.67 mM MgSO₄, 0.1 mM EDTA, 33.3 mM DTT, 270 μ M coenzyme A, 530 μ M ATP, 470 μ M d-luciferin, pH 7.8). After 3 min luminescence was measured for 1 sec with a Victor²-1420 Multilabel counter

(PerkinElmer). Prior to data-collecting functional assays, several tests were performed to ascertain the adequate time for cell lysis and incubation with luciferase buffer. Since both buffers did not contain ligands, diffusion from the receptor was possible. The ligand-induced changes in luciferase activity were stable for at least 10 min after cell lysis [see Additional file 6].

To estimate receptor surface expression, we used an indirect cellular ELISA [39]. Three days after transfection cells were fixed with 4% formaldehyde in PBS for 30 min at room temperature. After washing with PBS and blocking with 10% FBS in DMEM at 37°C for 1 h cells were incubated with biotinylated anti-HA-antibody (Roche, 1 μ g/ml in DMEM with 10% FBS) at room temperature for 2 h. Plates were washed and incubated with streptavidin-horse-radish peroxidase conjugate (Roche, 1:5000 dilution in DMEM with 10% FBS) at room temperature for 1 h, followed by extensive washing. Enzymatic reactions were carried out at room temperature in the presence of H₂O₂ and o-phenylenediamine. The reaction was stopped by adding 50 μ l of 50 mM Na₂SO₃ in 1 M HCl. Colour development was measured bichromatically at 492 nm and 620 nm using a Sunrise[™] plate reader (Tecan).

**Figure 4**

Allosteric modulation of enzyme activity in M_3R -luciferase fusion proteins by nanomolar concentrations of M_3R blockers. CCh (square), atropine (open circle), scopolamine (filled circle) and butylscopolamine (diamond) were applied at the indicated concentrations on COS-7 cells transfected with M_3R -luciferase (A) and construct #8 (B) and luciferase activity was determined. The luminescences without ligands were $178,680 \pm 18,171$ AU (M_3R -luciferase) and $121,578 \pm 12,845$ AU (construct #8). Enzyme activity of the individual constructs without ligands was set at 100%. All data are given as mean \pm S.E.M. of four independent experiments each performed in triplicate. Control experiments were carried out with COS-7 cells expressing luciferase alone and V_2R -luciferase [see Additional file 3].

A paired, two-tailed Student's t-test was used to detect significant differences in luciferase activity after ligand binding. The number of replicates is given in the respective legends of the figures and table.

Abbreviations

AU: arbitrary units; FBS: fetal bovine serum; CCh: carbachol; DMEM: Dulbecco's modified Eagle medium; ICL1-3: intracellular loops 1-3; IP: inositol phosphate; M_3R : M_3 muscarinic acetylcholine receptor; TMH: transmembrane helix; V_2R : V_2 vasopressin receptor.

Authors' contributions

DT carried out all functional assays and participated in the generation of mutants. DL participated in the generation of the mutants. RS participated in designing the mutants. TS conceived of the study, participated in the design and wrote the manuscript. All authors read and approved the final manuscript.

Additional material

Additional file 1

Description of control experiments. This file summarises all control experiments carried out.

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[<http://www.biomedcentral.com/content/supplementary/1472-6750-9-46-S1.pdf>]

Additional file 2

Luciferase can be integrated into other GPCR (e.g. V_2R) without losing G protein-coupling abilities. COS-7 cells were transfected with wild type V_2R and V_2R -luciferase. 48 h after transfection cells were incubated with 100 nM arginine-vasopressin (AVP). Cyclic AMP levels were determined using the non-radioactive cAMP-determination kit (AlphaScreening technology, PerkinElmer). The cAMP level (atmol/cell) of two independent experiments performed in triplicate is given (means \pm S.E.M.).

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[<http://www.biomedcentral.com/content/supplementary/1472-6750-9-46-S2.jpeg>]

Additional file 3

M_3R ligands have no effect on soluble luciferase and V_2R -luciferase fusion protein. COS-7 cells were transfected with luciferase (A) and V_2R -luciferase (B). Increasing concentrations of CCh (square), atropine (open circle), scopolamine (filled circle) and butylscopolamine (diamond) were applied and luciferase activity was determined as described under Methods. The enzyme activities without ligands were $1,905,212 \pm 172,463$ AU (luciferase), $182,120 \pm 18,188$ AU (V_2R -luciferase). Enzyme activity of the individual constructs without ligands was set 100%. All data are given as mean \pm S.E.M. of three independent experiments each performed in triplicate.

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Additional file 4

Modulation of enzyme activity in M₃R-luciferase fusion proteins by different receptor ligands. COS-7 cells transfected with M₃R-luci and construct #8 were stimulated with the indicated ligands (100 μM) and luciferase activity was determined. The luminescence of the fusion constructs were 184,314 ± 75,049 AU (M₃R-luci) and 140,452 ± 55,426 AU (construct #8). Enzyme activity of the individual constructs without ligands were set 100%. All data are given as mean ± S.E.M. of four independent experiments each performed in triplicate.

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Additional file 5

Primer used in this study. This table lists all primers used to generate the mutants of the M₃R-luciferase fusion protein.

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Additional file 6

Kinetics of luciferase activity assay. Cells were transfected with M₃R-luci and luciferase and the assay was performed as described in the Method section with the exception that the luciferase buffer was added at different incubation times after lysis. Data are given as mean ± S.D. of one experiment performed in triplicate.

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[<http://www.biomedcentral.com/content/supplementary/1472-6750-9-46-S6.jpeg>]

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